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MYCOFABRICATION AND CHARACTERIZATION OF SILVER NANOPARTICLES BY USING SOME ENDOPHYTIC FUNGI WITH SPECIAL REFERENCE TO THEIR ANTIMICROBIAL POTENTIAL

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ABSTRACT

In the present study a total of 106 endophytic fungi were isolated from 30 different medicinal plants. When subjected to silver nanoparticles synthesis protocol, 11 isolates gave positive results. Out of these silver nanoparticles synthesized by Alternaria tenuissima PGL#71 isolated from Punica granatum, exhibited maximum broad spectrum antimicrobial activity against test pathogenic microorganisms. Highest zone of inhibition was obtained against Escherichia coli (MTCC# 82) (32±0.82mm) followed by Bacillus subtilis (MTCC# 441), (28±0.41 mm) and Salmonella typhimurium (MTCC #3904) (28±0.28mm). Out of three fungal strains highest activity in terms of zone of inhibition was observed against Candida albicans (MML#25) (18±0.25 mm) which was followed by moderate activity against remaining two pathogenic fungal strains viz., Candida kruezi (MML#10) (10±0.23 mm) and Candida glabrata (MML#32) (6.63±0.22 mm). During UV-VIS Spectroscopic study of above sample a sharp Surface Plasmon Resonance peak was obtained confirming the synthesis of silver nanoparticles. Two peaks obtained durin Dynamic Light Scattering Study clearly indicates that silver nanoparticles synthesized in the present study have different sizes. The zeta potential analysis revealed that synthesized silver nanoparticles possess negative charge. Further the Fourier Transform Infrared spectrum confirmed presence of Carboxyl and Amine groups with silver nanoparticles. Finally, electron microscopic images showed the formation of spherical silver nanoparticles having dimensions ranging from 2nm to 20 nm.

KEYWORDS: Silver Nanoparticles, Antimicrobial activity, Biosynthesis, Eco Friendly, Green Synthesis & Biofactories

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Original Article

INTRODUCTION

Nanoparticles are particles having size in the range of 1-100 nm. Because of their extremely small size they exhibit certain unique properties which make them applicable in a variety of fields. Their uniqueness arises due to larger surface area to volume ratio, greater catalytic activity, high thermal and electrical conductivity and better tensile strength (Liu et al. 2009). They show promising applications in different field's viz., electronics, biological labelling, diagnostics, catalysis, optics and medicine. Recently, nanoparticles particularly silver nanoparticles have taken a centre stage in the pharmaceutical and biomedical sector because of the increasing antibiotic resistance problem amongst pathogenic microorganisms. This has attractive the attention of researchers towards finding novel ecofriendly approaches for nanoparticles synthesis. Endophytic fungi have evolved as potential biofactories for green synthesis of silver nanoparticles. Therefore present research work was planned to explore endophytic fungal flora for their silver nanoparticle synthesis potential and screening the antimicrobial activity of mycofabricated silver nanoparticles against test pathogenic microorganisms.

MATERIALS AND METHODS

Isolation of Endophytic Fungi

Healthy parts of some medicinal plants were collected and surface sterilized according to the method described by Strobel et al. (2003) with slight variation. These plant samples were washed under running tap water to remove dust particles and then sliced into small pieces of 2mm in size. All pieces were sequentially soaked in 70% ethanol, 4% sodium hypochlorite solution and 70% ethanol for 1min, 2min and 1min respectively. Finally they were rinsed in sterile distilled water and dried on sterilized Whatman filter paper No. 1. Dried plant pieces were then placed on freshly prepared Potato Dextrose Agar (Potato-200gms, Dextrose-20gms, Agar-20gms and distil water-1000ml) (PDA) plates and incubated at 28 ± 2^0C for 7 days. PDA plates containing pieces of plant parts were periodically observed for fungal growth. Fungi growing out from explants were then sub-cultured on PDA plates/slants and maintained in refrigerator at 4ºC (Rodrigues and Samuels, 1999).

Identification of Endophytic Fungi

Morphological Identification by Slide Culture Technique

All isolated fungal strains were identified on the basis of morphological characteristics by using slide culture technique. In this method Potato dextrose agar medium was poured on a sterilized glass plate kept in a sterilized moist chamber in the form of a thin film (upto 5mm). After solidification, the film was cut into small cubes with flamed scalpel. These cubes were placed on slides inside the moist chamber and inoculated with fungal spores separately. Inoculated slides were incubated at 28 ± 2^0C for 5 days. After sporulation one drop of cotton blue and lactophenol was poured (Agarwal & Hasija, 1986) and identification was done microscopically by comparing the morpho-taxonomic characteristics with those given in various literatures, monographs, bulletins, checklist, mycological journals and books.

Molecular Identification by ITS Sequencing and Blast Search

The most potent endophytic fungal strain was characterized at molecular level by using Internal Transcribed Spacer (ITS) Gene Sequencing followed by Blast search analysis. The molecular identification was completed under following steps:

- **Genomic DNA Isolation**

For isolation of Genomic DNA in pure form, the endophytic fungal isolate was grown in Potato dextrose broth under shaking conditions at 28ºC until the log-phase was achieved. This culture was then centrifuged at 6,000 × *g* for 15 min at 4°C. The pellet so obtained was transferred to 200 µl of extraction buffer (0.2 M Tris-HCl [pH 7.6], 0.5 M NaCl, 0.1% sodium dodecyl sulfate, 0.01 M EDTA) after washing twice with 0.8% physiological saline. This mixture was vortexed vigorously after adding Glass beads at a 1:1 ratio for cell lysis. DNA was extracted from this lysate by using a DNeasy plant mini kit (Qiagen, Hilden, Germany). Further analysis of DNA was performed via, electrophoresis on a 1% agarose gel in presence of 1× TBE buffer (8.9 mM Tris-borate, 0.2 mM EDTA) after staining with ethidium bromide. Finally, the extracted DNA was stored at -20ºC after UV-VIS Spectrophotometric analysis at 260 and 280 nm for checking its purity.

- **Amplification of ITS Region**

Amplification of purified DNA was done with a thermal cycler (Helena Biosciences), by using fungal universal primers ITS4 and ITS5 synthesized by Sigma Aldrich House. Reaction mixture (25-µl) was prepared by mixing 0.1 µM of each primer, 100 µM deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Qiagen), 1x PCR buffer with 2.0 mM $MgCl_2$ and 2 µl of template DNA sample. The reaction involved initial denaturation at 96°C for 10 min, followed by 30 cycles in series of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final step of one cycle at 72°C for 10 min to final extension. Products of reaction were analyzed and stored for further use. A reaction mixture without sample DNA was used as a negative.

- **Sequencing of PCR Products**

The amplified products obtained after PCR with primer pair ITS4 and ITS5 were then sequenced to determine the complete sequences. The whole process was performed in an ABI Prism Automated DNA Sequencer (model 3100, version 3.0; Applied Bio systems). The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit.

- **Editing of Amplified Sequences**

Manual editing of the raw sequence obtained from ABI 3100 Automated DNA Sequencer was conducted to remove inconsistency.

- **BLAST Analysis**

Finally the sequence data obtained was analyzed with the help of BLAST (www.ncbi.nlm.nih.gov/BLAST) program. During this analysis the sequence was aligned with publicly available sequences and identification of fungus was done on the basis of multiple-sequence alignments as determined with Clustal W program.

Screening of Endophytic Fungi for Extracellular Synthesis of Silver Nanoparticles

It was accomplished under following steps:

Biomass Preparation

All isolated endophytic fungal strains were grown in different flasks containing potato dextrose broth liquid medium (PDB) at 28±2°C for 96 h. 10gm biomass (wet weight) of each endophytic fungus was harvested by filtration through Whatman filter paper No.1, and then washed with sterilized Milli Q water to remove any components of the medium. These fungal mycelia mats were used for silver nanoparticle synthesis (Devi et al. 2015).

Biosynthesis of silver nanoparticles (Extracellular synthesis)

10 gms harvested biomass of each endophytic fungus was then immersed in 100ml sterilized Milli Q water and incubated at 28±2ºC for 72hrs. After incubation these suspensions were filtered through Whatman filter paper No. 1 and filtrates (Mycelia Free Water Extracts) so obtained were mixed with 1mM silver nitrate and incubated at 28±2ºC for 72 hrs. They were then examined regularly for reduction which is indicated by change in the colour of reaction mixture to yellowish brown followed by UV-VIS Spectroscopic analysis (Ninganagouda et al. 2013). Two controls, one containing Mycelia Free Water Extract of endophytic fungi without silver nitrate and other containing sterilized Milli Q water with 1mM silver nitrate solutions were used as standards.

Screening of Potential Fungal Strain for Biosynthesis of Silver Nanoparticles

On the basis of visual observation, reaction mixtures showing colour change from pale yellow to brown were screened out preliminarily and further examined under UV-Visible Spectrophotometer for the presence of characteristic Surface Plasmon Resonance peak exhibited by silver nanoparticles. Those endophytic fungal isolates whose reaction mixtures exhibited absorption peak within the range of characteristic absorption spectrum of silver nanoparticles were selected for further study.

Evaluation of Antimicrobial Activity of Silver Nanoparticles

In the present research work antimicrobial activity of myco-synthesized silver nanoparticles was tested by using the Agar Well Diffusion Assay (Norrel and Messely, 1997). In this method 80µl of diluted cultures of pathogenic bacterial and fungal strains were seeded on to the surface of different Nutrient Agar Media (NAM) and Sabrouds Dextrose Agar (SDA) plates respectively *via* spread plate technique. Wells of 4mm diameter were aseptically made in the seed agar and 20µl of silver nanoparticle solution was loaded into these wells. Plates containing bacterial cultures were then incubated at 37ºC for 24hrs and those containing fungal cultures were incubated at 28±2ºC for 48 hrs. Antimicrobial activities of silver nanoparticles samples were recorded in terms of diameter of inhibition zone, expressed in millimetre. The sample showing highest antimicrobial activity was chosen for further characterization studies. The fungus responsible for formation of these particles was considered to be the most potent fungal strain.

Determination of Minimum Inhibitory Concentration (MIC) of Prepared Silver Nanoparticles

In the present study MIC against test Bacterial strains (Minimum Bactericidal Concentration, MBC**)** as well as against the test Fungal strains (Minimum Fungicidal Concentration, MFC) was determined by Broth Dilution Method according to Clinical and Laboratory Standards Institute (CLSI) 2000 (Chandrakanth et al. 2014).

Bacterial cells were grown in 10 ml Luria Bertin (LB) broth by inoculating 10 µl of 18 hrs culture, supplemented with 2, 4, 6, 8, 10, 12, 14, and 16µg ml^{-1} of silver nanoparticles. All tubes were incubated at 37ºC for 18 hrs and absorbance was measured at 600 nm.

Similarly, fungal cells were grown in Sabrouds Dextrose Broth (SDB) by inoculating 10 µl of 24 hrs culture, supplemented with 2, 4, 6, 8, 10, 12, 14, and 16µg ml^{-1} of silver nanoparticles. All tubes were incubated at 28±2ºC for 24 hrs and absorbance was measured at 600 nm.

Characterization of Mycofabricated Silver Nanoparticles

UV-Visible Spectroscopy

Silver nanoparticles are known to show absorbance in the range of 390-444nm due to Surface Plasmon Resonance phenomenon. Therefore, absorption peaks obtained within this range confirms the presence of silver nanoparticles in the respective reaction mixture. It is considered to be a reliable and accurate analytical laboratory assessment procedure for the study of silver nanoparticles (Devi et al. 2012).

Formation of silver nanoparticles was preliminarily confirmed by visual observation of colour change from pale yellow to reddish brown after adding 1mM silver nitrate to the mycelia free water extract of *Alternaria tenuissima* PGL#71. It was further confirmed by sharp peak given by silver nanoparticles in the visible region using UV-Visible spectroscopy.

For absorbance measurments the colloidal brown solution was poured in quartz cuvettes and placed in sample holder. Wavelength range characteristic for silver nanoparticles was passed through the sample containing cuvettes. The absorbance value and absorption spectrum of silver nanoparticles were displayed in monitor and recorded. Mixture containing sterilized Milli Q water and 1mM silver nitrate was used as standard during the experiment.

Dynamic Light Scattering (DLS)

This technique is used to determine the size distribution profile of small particles in solution viz., proteins, polymers, micelles, carbohydrates, and nanoparticles. The silver nanoparticles present in the brown colloidal solution were analyzed by this technique to know their average size distribution. Samples were prepared either by centrifugation to remove dust and artifacts from the solution. The dried powdered was then diluted in sterilized Milli Q water and subjected to the light source. The information regarding size distribution was obtained in the form of a graph containing sharp peaks at specific wavelengths. The average value of peaks was calculated to determine average size distribution of silver nanoparticles present in the sample.

Zeta Potential

Zeta potential is widely used for quantification of the magnitude of charge which is a key indicator of the stability of colloidal dispersions. The magnitude of zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is small, attractive forces may exceed this repulsion and the dispersion may break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate. Therefore the colloidal silver nanoparticle solution was analyzed via Zeta Sizer instrument to measure their zeta potential and infer about their stability. The sample was poured into sample holders of the instrument and analyzed.

Field Emission Gun - Transmission Electron Microscopy (TEM)

It is also known as High Resolution Transmission Electron Microscopy (HRTEM). It is an imaging mode that allows the imaging of crystallographic structure of nanoscale materials (Joshi et al. 2008).

Characterization of silver nanoparticles was done by this technique to know the size, shape of a material in nano-dimension and to study the crystal structure meticulously. TEM is a microscopic technique wherein beam of electron is transmitted through an ultra thin specimen and interacts as electrons waves exciting from the sample to form an image. The samples were prepared by drop-coating silver nanoparticles solution onto the carbon-coated copper grid and kept under vacuum before loaded onto a specimen holder. TEM micrographs were taken and then sizes, shape and crystalline structure of silver nanoparticles were confirmed.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a chemical analytical technique which measures the infrared intensity versus wavelength (wave number) of light, which in turn detects chemical functional groups present in the sample. The main goal of IR spectroscopy is to determine the interaction between protein and silver nanoparticles which is responsible for reduction, capping and effective stabilization of silver nanoparticles.

The silver nanoparticles synthesized was centrifuged at 10,000 rpm for 15 mins and then the pellet formed was resuspended in distilled water and again centrifuged at 10,000 rpm for 10 min. Collected pellet was air dried at room temperature and subjected to FTIR analysis in the range of 500 to 4000 cm^{-1} diffuse reflectance mode.

Approximately 0.1 to 1.0 % sample (dried pellet prepared above) was well mixed into 200 to 250 mg fine alkali halide (KBr) powder and then finely pulverized and put into a pellet-forming die. A force of approximately 8 tons was applied under a vacuum of several mm Hg for several minutes to form transparent pellets. Degassing was performed to eliminate air and moisture from the KBr powder. Inadequate vacuum may result in easily broken pellets that scatter light.

RESULTS AND DISCUSSIONS

Isolation of Endophytic Fungi

A thorough survey of various sites of Jabalpur and nearby regions was conducted and total 30 plant samples were collected for isolation of endophytic fungi. A Total of 106 endophytic fungi were isolated and maintained in pure state on well labelled PDA slants in multiple copies at 4ºC. Several researchers have isolated endophytic fungi from different medicinal plants for synthesis of metal nanoparticles. Devi et al. (2015) isolated three endophytic fungi viz., *Aspergillus tamari* PFL2, *Aspergillus niger* PFR6 and *Penicillium ochrochloron* PFR8 from ethno-medicinal plant *Potentilla fulgens* L. and used them for biosynthesis of silver nanoparticles. Similarly, an endophytic fungus *Aspergillus clavatus* isolated from stem tissues of *Azadirecta indica* was used for the synthesis of silver nanoparticles (Verma et al. 2010).

Morphological Identification of Endophytic Fungal Isolates by Slide Culture Technique

On the basis of morphological characteristics, out of 106 fungal isolates 99 sporulating isolates were identified belonging to different genera viz., *Colletotrichum, Sclerotium, Fusarium, Curvularia, Alternaria, Botrytis and Phoma*. Whereas, 7 non-sporulaing isolates cannot be identified and hence were named as sterile mycelia**.** Likewise endophytic fungi viz., *Alternaria solani* (GS1) and *Penicillium fumiculsum* (GS2) isolated from ethno-medicinal plant *Glorisa superrba* were utilized for biosynthesis of silver nanoparticles (Devi et al. 2014). In an another study, endophytic fungus *Fusarium* sp. isolated from leaves of *Withania somnifera* (Ashwagandha) was identified morphologically and used for silver nanoparticle synthesis (Singh et al. 2015).

Screening of Endophytic Fungal Isolates for the Biosynthesis of Silver Nanoparticles

As depicted in Figure 1 it was found that out of 106 isolates in case of 11 isolates viz., *Fusarium oxysporum* EOL#07, *Aspergillus niger* SCL#12, *Fusarium solanii* EAL#27, *Colletotrichum* sp. AVL#30, *Alternaria alternate* OSL#33, *Phyllostica* sp. TPL#48, *Penicillium janthinellum* AML#55, *Aspergillus flavus* SAL#61, *Alternaria* sp. PGL#71, *Penicillium funiculosum* GSL#82 and *Aspergillus tamarii* BML#98, the colour of reaction mixtures changed from colourless to brown indicating reduction of silver nitrate to silver nanoparticles. Appearance of brown colour was due to the Surface Plasmon Resonance phenomenon exhibited by silver nanoparicles. This was further confirmed by analyzing brown reaction mixtures of all 11 endophytic fungal isolates via UV-Visible Spectrophototmeter wherein all brown reaction mixtures exhibited an absorbance peak falling in the range of characteristic absorption spectrum for silver nanoparticles (Figure 2).

Figure 1: Brown Coloured Reaction Mixtures of 11 Endophytic Fungal Isolates Obtained During Screening for Biosynthesis of Silver Nitrate

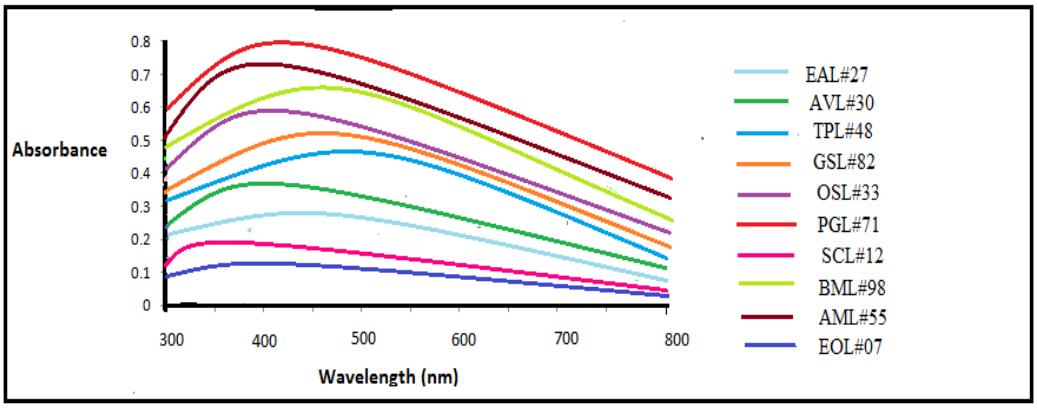


Figure 2: UV-VIS Absorption Spectrum of Brown Reaction Mixtures of 11 Endophytic Fungal Isolates

Screening of Biosynthesized Silver Nanoparticles for Their Antimicrobial Potential

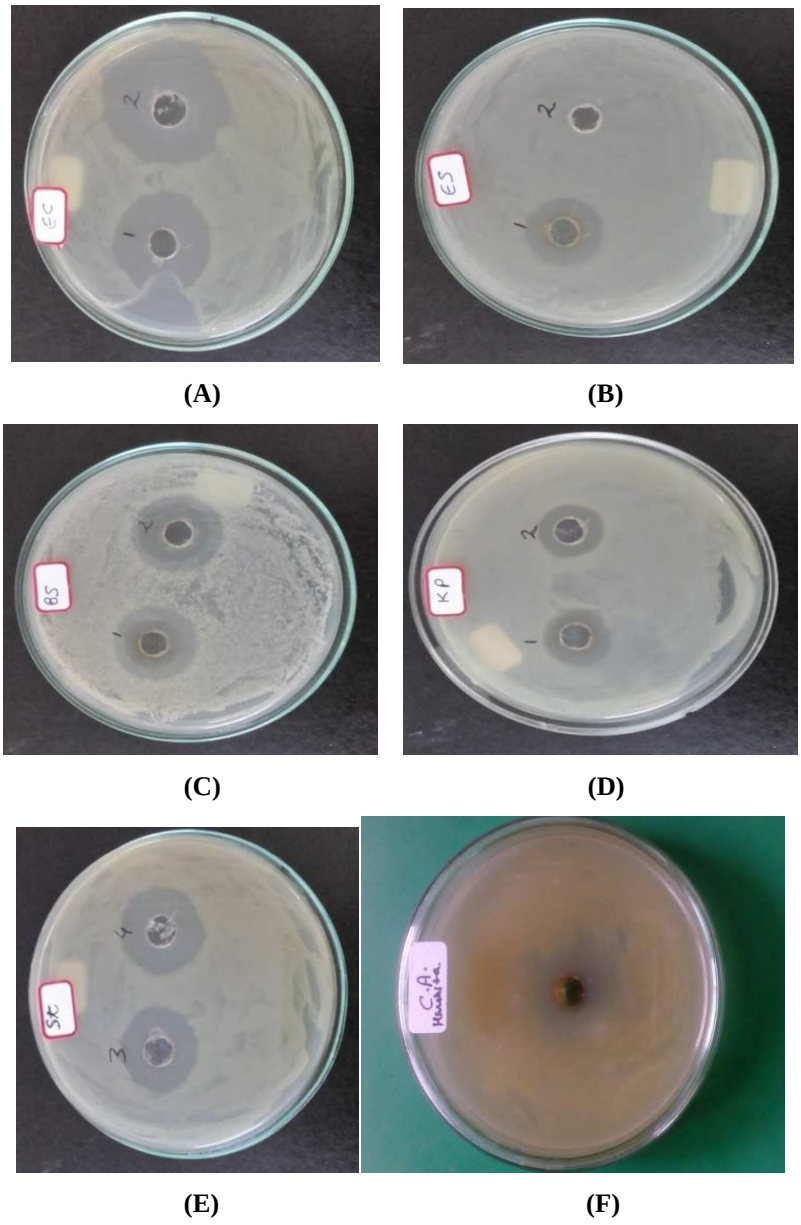
This was done by testing their antibacterial and antifungal activity against some pathogenic bacteria and fungi respectively *via* Agar Well Diffusion Assay.

Highest antimicrobial activity was observed in case of silver nanoparticles synthesized by the endophytic fungal isolate *Alternaria* sp. PGL#71 isolated from *Punica granatum*. Maximum zone of inhibition (Figure 3) was obtained against *Escherichia coli* (MTCC# 82) (32±0.82mm) followed by *Bacillus subtilis* (MTCC# 441), (28±0.41 mm) and *Salmonella typhimurium* (MTCC #3904) (28±0.28) (Figure 3). On the other hand it showed moderate activity against *Enterococcus* sp. (MTCC# 737) (16±0.82mm) and *Klebseilla pneumoniae* (MTCC #109) (16.6±0.29 mm). Besides pathogenic bacterial strains these silver nanoparticles also exhibited considerably good activity against test pathogenic fungal strains. Out of three fungal strains highest activity in terms of zone of inhibition was observed against *Candida albicans* (MML#25) (18±0.25 mm) which was followed by moderate activity against remaining two pathogenic fungal strains viz., *Candida kruezi* (MML#10) (10±0.23 mm) and *Candida glabrata* (MML#32) (6.63±0.22 mm).

Silver nanoparticles synthesized by *Fusarium oxsporum* EOL#07 followed *Alternaria* sp. PGL#71 and expressed good activity against four pathogenic test bacteria. However no activity was observed against one pathogenic bacterial strain and three fungal pathogens. On the other hand, remaining 9 silver nanoparticles samples were found to exhibit low activities against test bacterial strains and almost no activity against test fungal strains.

Therefore, endophytic fungal isolate *Alternaria* sp. PGL#71 was selected for further large scale synthesis of silver nanoparticles, optimization and characterization studies.

Variation in antimicrobial activities of silver nanoparticles has been recorded by various earlier workers. Verma et al. (2010) investigated the antimicrobial activity of silver nanoparticles synthesized by endophytic fungus *Aspergillus clavatus* against *Candida albicans* IMS/Ca-025, *Pseudomonas fluorescens* IMS/Pf-034 and *Escherichia coli* IMS/Ec-071. Devi et al. (2014) studied the antimicrobial activity of silver nanoparticles synthesized by using endophytic fungus *Penicillium* sp. isolated from medicinal plant *Centella asiatica.* These particles were found to produce inhibitory effect on bacterial pathogens like *Proteus mirabilis, Shigella dysentriae, Klebsiella pneumoniae, Staphylococcus aureus,* as well as against fungal pathogens such as *Candida albicans.*



(A) **(B)**

(C) **(D)**

(E) **(F)**

Figure 3: Antimicrobial Activity of Silver Nanoparticles Synthesized by *Alternaria tenuissima* PGL#71Against: (A) *Escherichia coli*, (B) *Enterococcus sp.*, (C) *Bacillus subtilis*, (D) *Klebseilla pneumonia*e, (E) *Salmonella typhimurium*, (F) *Candida albicans*

Molecular Identification of Potent Strain

The endophytic fungal isolate *Alternaria* sp. PGL#71 which synthesized silver nanoparticles possessing highest antimicrobial activity was characterized by 18S rRNA gene using ITS primers.

- The tested fungal culture *Alternaria* sp. PGL#71 showed **99%** sequence similarity with ***Alternaria tenuissima.***
- Sequence analyses with NCBI accession number KR611592, ***Alternaria tenuissima*** strain NKG1 resulted in following alignment statistics.
- Alignment statistics: Query Length - 449, Score - 807 bits (894), Expect - 0.0, Identities - 448/449 (99%), Gaps - 0/449 (0%), Strand - Plus/Minus

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Query  1
             AGGCCGGCTGCCAATTACTTTAAGGCGAGTCTCCAGCAAAGCTAGAGACAAGACGCCCAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  61    CACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGGAATACCA  120
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Query  121   AAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACT  180
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Query  181   TATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTT  240
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Query  361   CAAGGCTGTAACCCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGC  420
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Sbjct  102   CAAGGCTGTAACCCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCCTCCGC  43

Query  421   AGGTTCACCTACGGAGACCTTGTTACNCC  449
             |||||||||||||||||||||||||  ||
Sbjct  42    AGGTTCACCTACGGAGACCTTGTTACCCC  14
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Figure 4

Table 1: Top Five Hits upon BLAST Analysis

Gene Bank Accession No.	Description	Max Score	Query Cover	Query Coverage	E Value	Identity (%)
KR611592.1	*Alternaria tenuissima* strain NKG1	807	807	100%	0.0	99%
KC178638.1	*Alternaria* sp. MLM1	807	807	100%	0.0	99%
KC139468.1	*Alternaria* sp. YXM9	807	807	100%	0.0	99%
KU059951.1	*Alternaria alternata* strain 17.19CR2-530.1	805	805	99%	0.0	100%
KU059929.1	*Alternaria alternata* strain 17.2CR2-520.1	805	805	99%	0.0	100%

Netala et al. (2016) reported the synthesis of silver nanoparticles by using an endophytic fungus isolated from *Centella asiatica*. This fungus was identified as *Aspergillus versicolor* ENT7 based on 18S rRNA gene sequencing.

Determination of Minimum Inhibitory Concentration

It was determined by broth dilution method was used in the present study. According to data obtained the growth of *Escherichia coli* cells was inhibited at a concentration of 8µg/ml of silver nanoparticles. Hence it was assigned as the minimum inhibitory concentration of the silver nanoparticles for *E.coli*. Whereas the minimum inhibitory concentration of silver nanoparticles for *Bacillus subtilis* and *Salmonella typhi* was found to be 12µg/ml. *Enterococcus* sp. and *Klebseilla pneumoniae* exhibited greater resistance towards the silver nanoparticles, as a result of which higher amount of silver nanoparticles were required to inhibit their growth. Minimum inhibitory concentrations of silver nanoparticles for these bacterial strains were 14 µg/ml and 16 µg/ml respectively.

On the other hand fungal strains also showed good resistance against silver nanoparticles used. Minimum concentration of silver nanoparticles which inhibited growth of *Candida albicans* was 14µg/ml and hence was assigned as its MIC. However the minimum inhibitory concentration of silver nanoparticles against *Candida kruezi* and *Candida glabrata* was 16 µg/ml.

Characterization of Mycosynthesized Silver Nanoparticles

This is the most important step of present research work since it reveals certain characteristic features viz., size, shape, texture, morphology etc. of silver nanoparticles synthesized by the endophytic fungus *Alternaria tenuissima* PGL#71.

UV-Vis Spectroscopy Analysis

This technique is used to measure the absorption of reflectance of any molecule in the visible or ultra-violet range (Skoog et al. 2007). In the present work it was used for confirmation of silver nanoparticles in the brown colloidal solution obtained after challenging mycelia free water extract of the endophytic fungus *Alternaria tenuissima* PGL#71 with 1mM silver nitrate (pH 6) and incubating the same at 30˚C for 72. The brown colour change is due to the reduction of silver ions into silver nanoparticles which in turn exhibit Surface Plasmon Resonance (SPR) phenomenon. The colloidal brown solution obtained in present study gave a sharp absorption band at 418nm wavelength (Figure 4) which is characteristic for silver nanoparticles thus confirming the presence of same in the brown solution.

Verma et al. (2010) confirmed synthesis of silver nanoparticles by an endophytic fungus *Aspergillus clavatus* by obtaining a SPR peak at 415 nm via, UV-Vis spectrophotometer. Similar findings were reported by many researchers (Raheman et al. 2011). On the other hand silver nanoparticles synthesized by using endophytic fungus *Penicillium* sp. exhibited an intense peak at 425 nm during the UV-VIS Spectroscopic analysis (Singh et al. 2014).

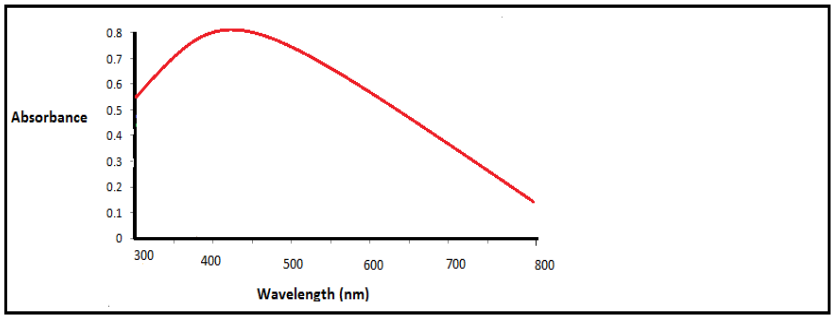


Figure 5: UV-VIS Absorption Spectrum of the Silver Nanoparicles Ample Synthesized under Optimized Conditions

Dynamic Light Scattering Analysis

It is a very important technique which is used to determine the size distribution pattern of very small particles present in a solution. The instrument used for DLS measurement is known as Zeta Sizer (Malvern - NanoZS 90). As shown in the Figure 5 two peaks were obtained at 8.6 nm and 37.8 nm respectively indicating that the sample prepared in the present investigation contains particles of different sizes ranging from 8 nm to 87 nm. The average size of these particles is 48.2 nm. Therefore silver nanoparticles prepared in this study are smaller than100 nm.

DLS measurements of synthesized silver nanoparticles for various concentrations of $AgNO_3$ were recorded and it was observed that the average particle size increases with the increasing concentration of silver nanoparticles i.e. 44.3 ± 11.6, 51.7 ± 16.2, 58.5 ± 20.9, 66.9 ± 28.5 and 70.1 ± 33.0 nm corresponding to the $1mgml^{-1}$, 5 mg ml^{-1}, $10mgml^{-1}$, $15mgml^{-1}$ and 20 mg ml^{-1} colloidal samples respectively (Shivnanda et al. 2016). In another study by Ramalingam et al. (2016) the average size of silver nanoparicles synthesized by endophytic fungus *Curvularia lunata* was revealed to be 64.3 nm.

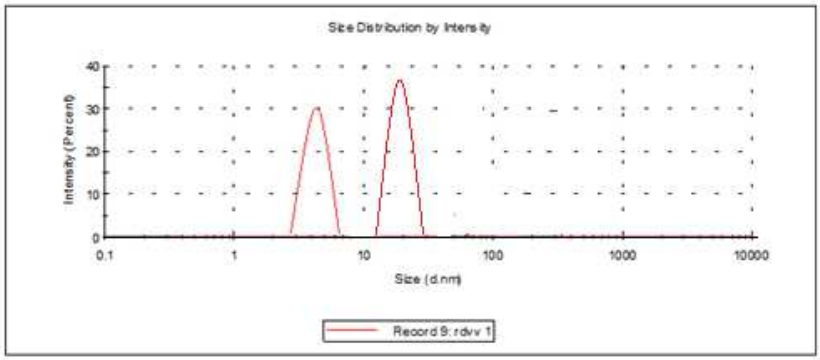


Figure 6: Size Distribution Pattern of Silver Nanoparticles by Dynamic Light Scattering

Zeta Potential Analysis

Due to their extremely small size nanoparticles are highly energetic, this makes them unstable. Therefore particles undergo agglomeration/aggregation to stabilize themselves and also develop certain charges on their surface which contributes to their stabilization. These charge potential are measured by using the zeta sizer (Malvern – Nano ZS 90). Zeta potential (Surface potential) has direct relation with the stability of a form/structure. Figure 6 indicates that the zeta potential of silver naopartciles synthesized in the present research work is -0.107 mV.

On the other hand zeta potential of pure silver nitrate as shown in Figure 7 is -13.1 mV. This change in the zeta potential of silver nitrate confirms its reduction into nanoparticles. Silver nanoparticles synthesized by endophytic fungus *Alternaria tenuissima* PGL#71have negative zeta potential. According to several previous reports generally silver nanoparticles possess negative charge (Gou et al., 2015).

Zeta potential of silver nanoparticles synthesized by using aqueous silk fibroin obtained from *Bombyx mori* was -33.6 mV (Shivananda et al. 2016). In a similar study by Ramalingam et al. (2015) the zeta potential of silver nanoparticles synthesized by an endophytic fungus *Curvularia lunata* was found to be -26.6 mV.

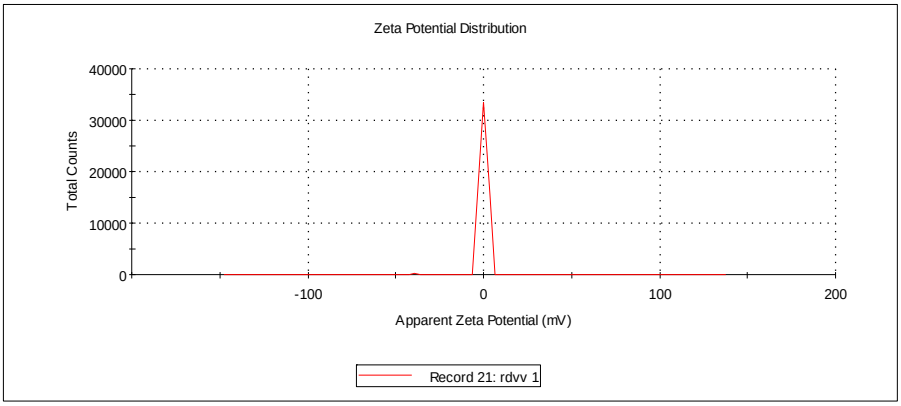


Figure 7: Zeta Potential of the Silver Nanoparticles Synthesized by *Alternaria tenuissima* PGL#71

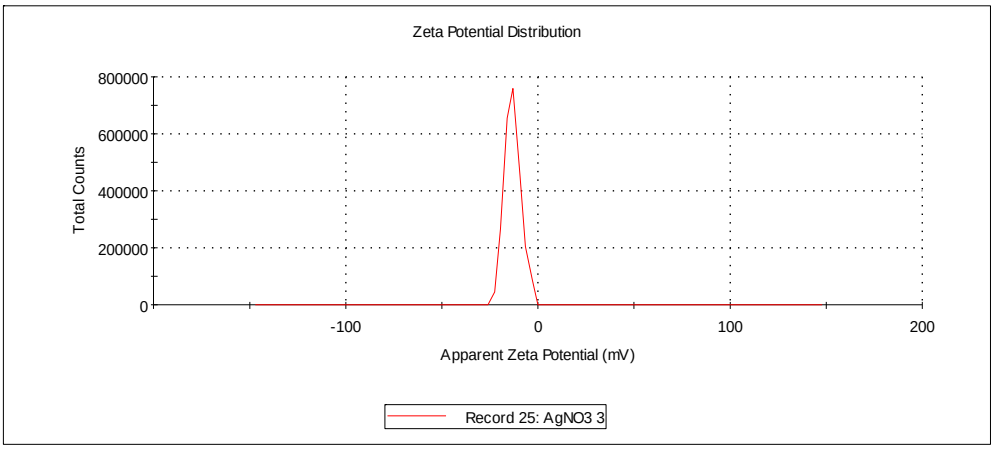


Figure 8: Zeta Potential of the Native Silver Nitrate

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

In this study FTIR measurements were carried out to identify the possible biomolecules responsible for reduction of Ag^+ ions and capping of metal nanoparticles for their efficient stabilization. All measurements were done by using FTIR Spectrophotometer Shimadzu 8400. The FTIR spectrum of silver nanoparticles synthesized by the endophytic fungus *Aternaria tenuissima* PGL#71 showed absorption peaks (Figure 8) corresponding to various functional groups viz., 1643 cm^{-1} denotes C=O and N-H stretching in primary amides, 3321 cm^{-1} represents $–NH_2$ stretching in aromatic amines, primary amines or amides, 2945 cm^{-1} indicates the presence of $–CH_3$ and $–CH_2$ stretching, 3541 cm^{-1} represents amide linkages. Data generated from FTIR analysis clearly indicates the presence of carboxyl (C=O) and amine (N-H) groups in the mycelia free water extract of *Alternaria tenuissima* PGL#71 which are probably involved in the reduction of Ag^+ ions to silver nanoparticles.

Several researchers have used FTIR analysis for confirmation of the presence of proteins and other biological compounds responsible for the synthesis and stabilization of silver nanoparticles (Daima et al. 2013; Venkatesan et al. 2014; Saxena et al. 2016).

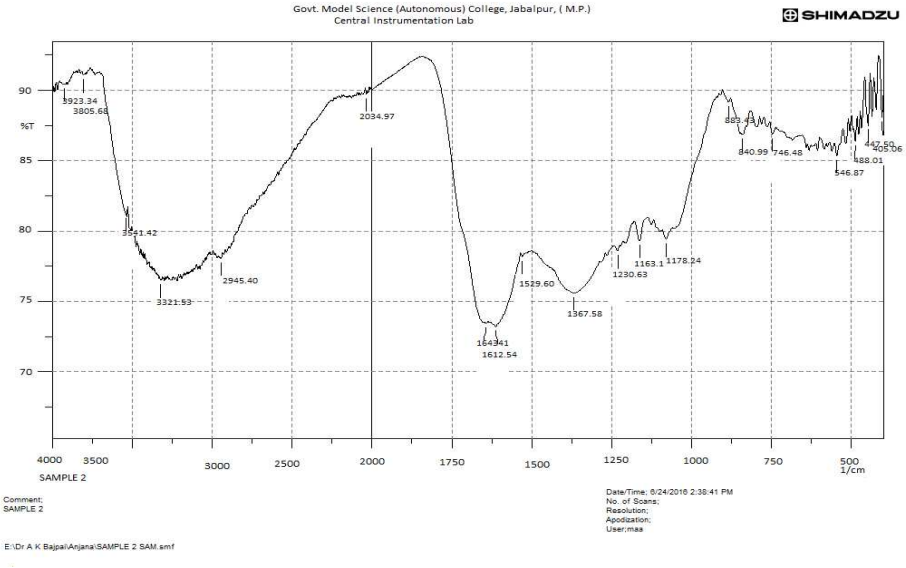


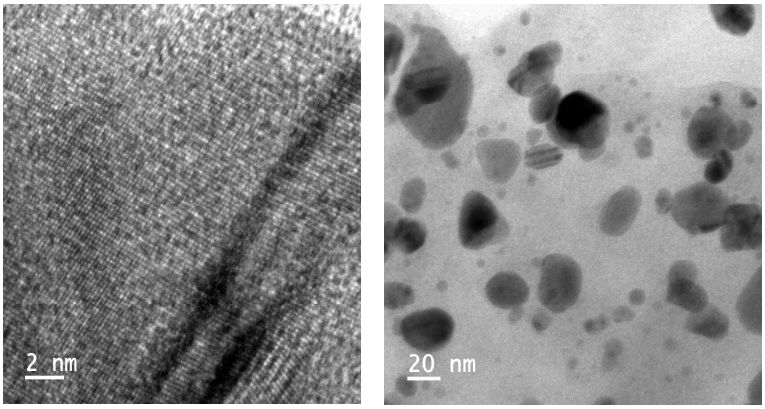
Figure 9: FTIR Spectrum of the Silver Nanoparticles sample synthesized by Alternaria tenuissima PGL#71

Field Emission Gun - Transmision Electron Microscopic (TEM) Analysis

The sample of silver nanoparticles prepared by *Alternaria tenuissima* PGL#71 under optimized conditions were studied by Field Emission Gun-Transmission Electron Microscope at the Indian Institute of Technology, Bombay. For analysis silver nanoparticles were deposited in the form of a thin film on carbon coated copper TEM grid and images of these particles were then captured. Images depicting (Figure 9) particles of 2nm and 20nm were obtained indicating the presence of particles ranging from 2-20 nm size. Besides, the TEM analysis revealed that synthesized silver nanoparticles possess variable morphology with most of them being spherical and poly-dispersed.

Verma et al. (2010) analyzed silver nanoparticles synthesized by endophytic fungus *Aspergillus clavatus* via Transmission Electron Microscopy and found particles ranging from 10-25 nm size.

Similarly, the size of silver nanoparticles synthesized by endophytic fungus *Penicillium* sp. as determined via Transmission Electron Microscope was from 25 to 30 nm (Rathod et al. 2014).



(a) **(b)**

Figure 10: Images of Silver Nanoparticles Synthesized by *Alternaria tenuissima* PGL#71 via., Field Emission Gun Transmission Electron Microscopy: a) 2nm Size Particles; b) 20 nm Size Particles

CONCLUSIONS

Biosynthesis of nanoparticles provides an attractive alternative for the hazardous chemical and physical methods of nanoparticles synthesis. The use of fungal cells has recently emerged as a novel approach for the synthesis of metal nanoparticles. Advantages of this method include tightly controlled and highly reproducible synthesis, ease with which the process can be scaled up, economic viability, tolerance towards high metal nanoparticle concentration in the medium, the generation of water-soluble, biocompatible particles and the avoidance of toxic surfactants or organic solvents. Compared to bacterial broth, fungal broth can be easily filtered by filter press or similar commonly used equipments, thus saving considerable investment costs for specialized equipments which may be needed for other methods. As a result, for large-scale production of nanoparticles use of fungi is preferred over other methods. While a number of reports are available on the biological synthesis of nanoparticles, the potential of endophytic fungi has still not been explored completely. Endophytic fungus *Colletotrichum* sp. isolated from geranium leave, *Aspergillus clavatus* isolated from sterilized stem tissues of *Azadirachta indica*, *Pestalotia* sp. isolated from leaves of *Syzygium cumini* are some examples of recently used fungi for the synthesis of nanoparticles with antimicrobial properties.

Out of all kinds of nanoparticles, metallic nanoparticles, including gold, silver, iron, zinc and metal oxide nanoparticles, have shown great promise in terms of biomedical applications, not only due to their large surface area to volume ratio, but also because they exhibit different biomedical activities. Silver nanoparticles are undoubtedly the most widely used nanomaterials among all. The use of silver in therapeutic approaches is well known since many years. Besides this they are also used in textile industries, water treatment, cosmetics industry etc. Biomedical applications of biosynthesised nanoparticles include wound healing properties, antimicrobial properties, anti-inflammatory properties etc. Moreover application of silver nanoparticles in drug delivery systems and diagnostic purpose has attracted the attention of researchers worldwide.

It is clear from the present research work that endophytic fungi isolated from various plants synthesize silver nanoparticles of varying size and morphology. These nanoparticles also show promising antimicrobial properties and therefore can be employed as Nanomedicines for the treatment of various diseases. Since, not much work has been carried out in this area, endophytic fungi needs to be explored more as potential biofactories for the green synthesis of silver nanoparticles.

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